Bioprospecting the Antimicrobial, Antiamoebic Activity and Phytoconstituents Evaluation of *Cyperus Rotundus* L.

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1. INTRODUCTION

Mankind has been depending on plants and its products from ancient times, for health care needs in particular, and various other reasons. Due to the introduction of chemotherapeutic agents, this dependence on herbal medicines has diminished **(Gera *et al*., 2018).**

Medicinal plants are a blessing of nature to human and have been used for ages to cure a good number of diseases. In many parts of the world, medicinal plants are used against bacterial, viral and fungal infections. Recently, the evaluation of plants which possess efficiency in curing various diseases is increasing **(Premlata *et al*., 2012)**. Infectious diseases have increased to a great extent during the recent years **(Mothana and Lindequist 2005)**, and they are the second leading cause of death across the world and the third leading cause of death of economically developed countries **(Luqman *et al*., 2005)**.Due to unselective use of commercial antimicrobials, the increase in resistance of multiple drug has slowed down the development of new synthetic antimicrobial drugs, and has imposed the search for new antimicrobials from other alternative sources **(Kunle *et al*., 2012; Neelam and Padma, 2008)**. Thus, there is progressively justified notion which claims that medicines derived from plants arecheaper and more active than contemporary medicine.

The studies of medicinal plants used as folklore medications have consequently attracted enormous attention of researchers in the scientific world in an effort to find possible solutions to the problems of multiple resistances to the existing synthetic and conventional antimicrobials **(Abbas *et al*., 2017; Van Whyk 2002; Teklehaymano *et al*., 2007)**.Recently, studies have exposed the presence of powerful healing agents: the phytochemicals in diversity of plants. Traditionally used medicinal and aromatic plants contribute a broad spectrum of biologically active compounds of known therapeutic activities **(Neelam and Padma, 2008)**

According to world health organization (WHO), 80% of population in developing countries depend on medicinal plants for their primary health care. India is one of the hotspots of biodiversity for medicinal plants in the world. Medicinal plant produces large amount of secondary metabolites which are helpful for human health care and maintain resistance against infection. A number of medicinal plants showed effective antimicrobial activities which were comparable to synthetic standard drugs.

World health organization estimate that the protozoan *Entameoba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually. Intestinal ameobiasis due to the infection of *E. histolytica* is ranked third on the list of parasitic protozoan infections leading to death behind malaria and schistosomiasis**. (Kabbashi *et.al*., 2015)**

Aim of this study is to estimate the antimicrobial and *in vitro* antiameobic activity of Cyperus *rotundus* L. *Cyperus rotundus* L. (Family: Cyperaceae) is a multivalent plant widely used in traditional medicine around the world for treatment of various diseases. This plant is also known as purple nutsedge or nutgrass (English), Motha (Hindi) or Musta(Sanskrit). *C. rotundus* is held esteem in Ayurveda, especially as a cure for gastrointestinal and joint ailments, this herb has been given special recognition in Ayurveda due to its multifaceted therapeutic benefits to cure various diseases. The studies conducted on animals and human subjects have shown that this widely

growing medicinal plants has several pharmacological activities including anti-inflammatory, antidiabetic, anti-diarrhoeal, antipyretic, analgesic activities etc. **(N. Singh *et .al*., 2012)**

2. REVIEW OF LITERATURE

Kabbashi, *et.al.,* (2015) have screened C. *rotundus* for antimicrobial activity against two Gram positive bacteria (*B. subtilis*, *S. aureus*), two Gram negative bacteria (*E. coli*, *P. aeruginosa*) as well as two fungi namely (*A. niger* and *C. albicans*) using the cup plate agar diffusion method. The extracts of whole plant of *C. rotundus* dissolved in DMSO (1:10) showed high activity (31 & 30 mm) against Gram positive bacteria (*S. aureus* & *B. Subtilis*) and (20 & 26 mm) against (*A. niger* & *C. albicans*). It also showed (19 & 20) against Gram negative bacteria (*E. coli* & *P. aeruginosa*). Therefore, this result showed that the extracts tested inhibited the growth of all microorganisms though the sensitivities of microorganisms varied.

Koko, *et. al*., (2015) have sreened the antiamoebic potential of the ethanolic extract of *C.rotundus* (whole plant), with different concentrations (500, 250 and 125 ppm) and Mertronidazole (the reference control) with concentration (312.5 µg/ml) was investigated against *E. histolytica* trophozoites *in vitro*. However, the ethanol extract of *C. rotundus* (whole plant) showed 100% inhibition at concentration 500 µg/ml after 96 h; which was compared with Metronidazole giving 96% inhibition at concentration 312.5 µg/ml at the same time against *E. histolytica.*

Khalid Karzan *et.al*., (2017) have screened Extracts of rhizomes-tubers, leave and oil of *Cyperus rotundus* plant were applied as antibacterial and antifungal agent against six pathogenic isolated bacteria and three species of fungi. *C. rotundus* is estimated as a promised plant, due to the presence of antimicrobial activity in many diseases caused by micoorganisms, such as; stomach, diarrhoea, dysentery and skin (Him-Che, 1985; Bown, 1995). *C. rotundus* in aqueous and ethanol extracts was used in randomly growing microbes on media enriched by 1% of extract, and agar well diffusion method by inoculating 0.4ml/well.

According to growth of targeted microorganisms on enriched medium by plant extract, both aqueous and ethanolic extracts of rhizomes-tubers had antibacterial activity on *E. coli* and *S. aureus*, while only ethanol extract exhibited anti-fungal activity against *A. niger*. In case of leave extract, only *E. coli* was susceptible to aqueous extract, but ethanolic extract had no effect on microorganisms. Furthermore, *C. rotundus* oil had antifungal potential against *C. albicans*, and no antimicrobial activity to others.

Agbo *et.al*., (2021) studied that the methanol extract showed the best activity against the test bacterial and fungal pathogens as it showed most significant inhibition at all concentrations. The leaf extracts at a concentration as low as 6.25mg/mL can significantly inhibit against both bacterial and fungal pathogens. At MBC and MFC of 6.25 mg/mL and 12.5 mg/mL, the plant extracts showed effective bactericidal and fungicidal effect against the test microbes.

Nasar *et. al*., (2015) studied the Essential oil of *Cyperus leavigatus* was extracted by hydroid stillation technique. The yield of the EO was 0.1 % of the total weight of the *C.leavigatus* aerial parts. The obtained volatile oil of *C. leavigatus* aerial parts was analyzed by gas chromatography-mass spectroscopy (GC-MS) Sixty-six compounds were identified. The constituents of the oil were identified by comparison with Kovat index with those obtained from the NIST/NBS libraries spectra. The results showed that EO composition include two major classes of compounds, terpenoids and hydrocarbones. Terpenes represent the major constituents 77.78 % including monoterpenes (47.6%), sesquiterpenes (21.49 %), and diterpenes derivative (8.36 %).

The antimicrobial and MIC assays were performed by the agar disc diffusion method.The results obtained by measuring the zone of inhibition diameter (mm). The first concentration not to produce visible inhibition zone after incubation was considered to be the MIC. The oil extract showed moderate antimicrobial activity against gram positive bacteria and yeast with no inhibition against gram negative bacteria and fungi.The ethyl acetate and dichloromethane extracts revealed strong antimicrobial activities against gram positive bacteria and a moderate antimicrobial activity against the gram negative bacteria in comparison with the positive controls thiamphenicol and nystatin. The total and methanol extracts showed a moderate antimicrobial activity against gram positive bacteria and fungi with no inhibition against gram negative bacteria and yeast.

3. OBJECTIVES OF THE STUDY

1. Collection, Identification, authentication and of *Cyperus rotundus* L.(Motha).
2. Phytochemical characterization of *Cyperus rotundus* L. by using HPTLC and GC-MS.
3. To study *in vitro* antimicrobial effect of phytoconstituents on some selected microbes.
4. To study *in vitro* Antiamoebic activity of isolated ethanol extract of *C. rotundus*
5. Calculation of MIC, MBC and MFC of isolated phytoconstituents.
6. To study the cytotoxicity assay of plant extract.

4. PLANT MATERIAL AND EXTRACT PREPARATION:

4.1. Plant Material and Extract Preparation

4.1.1. Collection of Plant Material

The native medicinal plant (botanicals) *C. rotundus* will be collected from in and around Nanded region to evaluate their antimicrobial and Antiamoebic activity. Plant will be screened on the basis of presence of antimicrobial and Antiamoebic properties according to literature or traditional knowledge, easy availability in bulk and having very less commercial value. Leaves, rhizome, seeds, etc. will be collected from in and around Nanded region. All healthy plant samples will be stored in plastic bags and brought back to laboratory. Plant will be thoroughly washed in running tap water; both fresh healthy materials and shade dried materials will be used for further work.

4.2. Cleaning and Sterilization

4.2.1. Surface Sterilization of Plant Material

The collected plant material will be thoroughly washed under the running taps water followed by surface sterilization with 1% Ethanol/H2O2and then will be washed with autoclaved distilled water. The surface sterilized plant material will then be dried in oven at 40^0 C for 5 days or until they will be dried completely.

4.2.2. Cleaning and Sterilization of Glasswares and Media

All the glassware required for research work will be kept in cleaning for overnight. Cleaning solutions will be prepared by dissolving 60g of potassium dichromate ($K_2Cr_2O_7$),60ml of concentrated sulphuric acids (Conc. H_2SO_4) in one litre of water. Each of these chemicals will be

dissolved separately in 500ml of water and finally mixed. All the materials to be used will be washed thoroughly with sterile distilled water finally mixed. All the materials to be used will be dissolved separately in 50 ml of water and finally mixed. All the materials to be used will be washed thoroughly with sterile distilled water and then dried in hot air oven at 160^0C for 60 min. **(Yenjerappa, 2009; Basamma,2013**).

The cleaned and dried petriplates will be wrapped with paper and sterilization in autoclaves.Test tubes and flasks having culture media will be plugged with non-absorbent cotton. These are covered with paper and futher autoclaved at 121^0C at 15 PSI for 20min. The nichromes wire loops, blades, scalpel, forceps will also be dipped in ethanol and then sterilized by heating on the flame.

4.3. Test Microorganism and Inoculum Preparation

Refference strain of bacteria and fungi will be used in the study will obtained from Microbial Type Culture Collection (MTCC) of Institute of Microbial Technology (IMTECH), Chandigarh (India). Following Microorganisms will be used for study:

Bacteria:

Gram positive bacteria- *staphylococcus aureus* and *Bacillus subtilis*

Gram negative bacteria- *Eschernia coli and Pseudomonas aeruginosa*.

Fungi: *Aspergillusniger and Candida albicans, Candida tropicalis.*

All bacteria will be cultured in nutrient agar medium at 37^0C for 24 hrs prior to use while the fungus will be cultured on potato dextrose agar at 28^0 C for 72 hrs. then suspended in potato dextrose broth to use.

Parasite isolate

Parasite isolate E. histolytica used in all experiments will be taken from patients of Hospitals Nanded. All parasite samples will be examined by wet amount preparation; the positive samples were transported to the laboratory in nutrient broth medium. Trophozoites of E. histolytica were maintained in RPMI 1640 medium containing 5% bovine serum at 37 ± 1°C.

4.4 ORGANIC EXTRACT PREPARATION AND THEIR ANTIMICROBIAL SCREENING

Different organic solvent such as hexane, ethyl acetate and methanol will be screened to work out the best extractant. Extraction will be carried out for the whole plant of C. rotundus plant by using overnight maceration techniques according to the method described in Harbone [7]. About 50 g round material will be macerated in 250 ml of particular organic solvent for 3 h at room temperature. Occasional shaking for 24 h at room temperature will be performed and, the supernatant will be decanted. Thereafter, the supernatant will be filtered under reduced pressure by rotary evaporation at 55°C. Each residue will be weighed and the yield percentage will be

calculated and then stored at 4°C in tightly sealed glass vial ready for use. The remaining extracts which is not soluble will be successively extracted using ethanol with the described technique. The extracts will be kept in freeze-dryer for 48 h, (Virtis, USA) until they will be completely dried. The residue will be weighed and the yield percentage will be calculated. The extracts will be kept and stored at 4°C until required.

4.4.1. *In vitro* testing of extracts for antimicrobial activity

The cup-plate agar diffusion method described in Kavanagh, [8] will be used adopted with some minor modifications to assess the antibacterial activity of the prepared extracts. One ml of the standardized bacterial stock suspension (between 108 and 109 CFU/ml) will be thoroughly mixed with 100 ml of molten sterile nutrient agar which will be maintained at 45°C. 20 ml aliquots of the inoculated nutrient agar will be distributed into sterile Petri-dish plates. The agar will be left to set and in all of these plates 4 cups (10 mm in diameter) will be cut using a sterile cork borer (No. 4) and agar discs were removed. Each cups were filled with 0.1 ml sample of the ethanolic extracts using an automatic microlitre pipette, and thereafter the extracts will be allowed to diffuse at room temperature for two hours. The plates were then incubated in an upright position at 37°C for 18 h. Two replicates will be carried out for each extract against each of the test organisms. After incubation the diameters of the resultant growth inhibition zones will be measured and averaged. The mean values will be tabulated.

Antifungal testing: The same method used for the antibacterial test was employed. However, the growth media used in case of fungi, was Sabouraud dextrose agar instead of nutrient agar. The inoculated medium was incubated at 25°C for two days for *Candida albicans* and three days for *Aspergillusniger.*

4.4.2. *In vitro* testing of extract for antiameobic activity

In vitro susceptibility assays used the sub-culture method CedilloRivera et al. (2002), which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *E. histolytica, Gairdia intestinalis* and *Trichomonas vaginalis* (Arguello-Garcia et al., 2004). 5 mg from each extract and compound will be dissolved in 50 µl of dimethyl sulfoxzide (DMSO) at Eppendorf tube containing 950 µl D.W in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates will be stored at -20°C for further analysis. Sterile 96- well microtitre plate was used for different plant extracts, positive control and negative control. Three columns of a microtitre plate wells [8 columns (C) × 12 rows (R)] will

bechosen for each extract, 40 µl) of an extract solution (5 mg/ml) will be added to the first column wells C-1: On the other hand, 20µl of complete RPMI medium will be added to the other wells of the second column and third column (C-2 and C-3). Serial dilutions of the extract will be obtained by taking 20 µl of extract to the second column wells and taking 20 µl out of the complete solution in C-2 wells to C-3 wells and discarding 20 µl from the total solution of C-3 to the remaining 20 µl serial solutions in the successive columns. 80 µl of culture medium will complemented with parasite and added to all wells. The final volume in the wells was 100 µl. In each test, Metronidazole (a trichomonocide) pure compound [(1-(2-hydroxyethl)-2-methyl-5 Nitroimidazole], was used as positive control in concentration 312.5 µg/ml, whereas untreated cells will be used as a negative controls (culture medium plus trophozoites). For counting, the samples were mixed with Trypan blue in equal volume. The final number of parasites was determined with haemocytometer three times for counting after 24, 48, 72 and 96 h. The mortality % of parasite for each extracts activity will be carried out according to the following formula

$$\text{Mortality of parasites (\%)} = \frac{\text{Negative control} - \text{tested sample with extract}}{\text{Negative control}}$$

5.MINIMUN INHIBITORY CONCENTRATION (MIC) STUDIES

A) Determination of Minimum Inhibitory Concentration (MIC)

The nutrient agar was prepared and sterilized ,then poured into sterile petr- idishes and allowed to solidify .the surface of the medium was inoculated with the test isolates .the discs soaked in different concentrations of each extract (100 mg/ml,50 mg /ml,25mg /ml,12.5 mg /ml and 6.25 mg /ml) wera placed on the surface of seeded nutrient agar .the plates will be incubated at 37^0C for 24 hrs ,after which they will be examined for the presence of growth inhibition ,the MIC was taken as the lowest concentration that prevent the growth of test organism .

B) Determination Of Miniumbactericidal Concentration (MBC) and Minium Fungicidal Concentrations (MBF)

A loopful of the content of each plate in the MIC determination which did not show any visible growth after the period of incubation was streaked into freshly prepared nutrient agar, to determined their MBC and then incubated at 37^0C for 24 hrs after which it was observed for visible growth the lowest concentrations of the subculture with no growth was considered as the minimum bactericidal concentration.this was carried out in triplicates and mean result will be taken .

6.QUALITATIVE AND QUANTITATIVE PHYTOCHEMICAL ANALYSIS

6.1. Phytochemical Test

Fresh stock solution of each extract will be prepared for each experiment. To prepare 100 mg/ml extract ,1g of either of the extract will be dissolved in 10 ml of distilled water, shaken vigorously to obtain a homogenous solution except for the glycoside test, for which 100 mg extract will be directly used. The procedures will be used earlierly described by Harborne (1973) and sofawora (1993).

6.2. Detection of the probable bioactive components.

In order to find out the component responsible for antifungal activity for the most active phytoconstituent, various techiniques such HPTLC (High Performance Thin Layer Chromatography), Gas chromatography-Mass spectrometry (GC-MS) and FTIR analysis will be performed.

6.3. High-Performance Thin Layer Chromatography (HPTLC)Analysis

HPTLC(high performance thin layer chromatography) fingerprinting of plant extracts will be carried out as per the method described by Ganeshan et al.,(2015).

6.4. Gas Chromatography –Mass Spectrometry (GC-MS) analysis

The GC-MC analysis of the most active band /s will be carried out and the mass spectrums of unknown compounds will be compared with the spectrums o**f** known compounds which was stored in the NIST and Wiley library .Interpritation of mass spectrum of GCMS was carried out by using data base present in NIST library having more than 62000 patterns. The molecular formula , molecular weight and name of the compounds were then ascertained (kiruthika and sornaraj,2011).

6.5. Fourier transfer infrared (FT-IR) analysis

Different stretching and bending vibrations of functional groups present in the medicinal plants under study will be monitored by FT-IR spectroscopy.

7. IMPORTANCE AND SIGNIFICANCE OF THE STUDY:

A) International status:

The World Health Organization (WHO) estimates that the protozoan *Entamoeba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually (WHO,1997**; Ravdin and Stauffer,2005**). Intestinal amoebiasis due to the infection of *E. histolytica* is ranked third on the list of parasitic protozoan infections leading to death behind malaria and schistosomiasis (Farthing et al., 1996). This infection remains a significant cause of morbidity and mortality world-wide (Stanley and Reed,2001). Amoebiasis is a rare occurrence in developed countries of the world, but only found in travelers, immigrants, homosexuals and institutionalized persons. *E*. *histolytica*-associated dysentery is a common occurrence in the less developed and developing countries of the world, but is more common in areas of low socioeconomic status, poor sanitation and nutrition especially in the tropics (**Ravdin and Stauffer, 2005**). Thus the majority of *E*. *histolytica* infections, morbidity and mortality occur in

Africa, Central and South America and the Indian sub-continent (**Haque *et al.,* 2000**). Metronidazole is the drug now widely used and recommended in the treatment of amoebiasis (**Townson *et al.*, 1994**). But it is less effective in the tissue than in the gut lumen (**Bhopale *et al.,* 1995**). In addition, it can eradicate only up to 50% of laminae infections (**Tierney etal., 1998**). Metronidazole sometimes causes adverse effects, example, myoplasia, neuralgia, and allergic dermatitis (**Upcrof *et al.*, 2006**). Hence, the present study will conducted to investigate the antiamoebic activity and cytotoxicity of *C. rotundus* (whole plant) in Maharashtra (Nanded).

D) National status :

Nowadays there is increasing interest of researchers to explore herbal drugs for health benefits of human as well as animals. Drugs which are obtained from natural sources are pharmacologically potent and have low or no side effects for use in preventive medicine. *Cyperus rotundus* L. is commonly known as nagarmotha, motha in local languages. It is available all over the country.

Cyperus rotundus is having some reported activities like antidiabetic, hypolipidemic, hepatoprotective and antimicrobial properties. It contains flavonoids, tannins, glycosides, monoterpenes, sesquiterpenes, sitosterol, alkaloids saponins, terpenoids, essential oils, starch, carbohydrates, protein, separated amino acids and different secondary metabolites. Considering the growing interest in the field of plant drugs assessing different pharmacological activities. In this study we are discussing about the antimicrobial activity and antiamoebic activity as well as cytotoxicity assay of *C.rotundus.*

C) Regional status:

The huge research gap has been came forth according to review of literature and this study lead some of the unnoticed medicinal values which have been practiced local.

D)Significance of the study:

A major challenge in global health care is the need for novel, effective and affordable medicines to treat microbial infections. therefore, the development of an alternative drug lines of treatment for infectious diseases is of ulmost importance. Plant have an amazing ability to produce a wide variety of secondary metabolites, like sesquiterpenes, flavonoids, saponins, alkaloids, phenolic acids, coumarins and steroids etc..some of the natural phytochemicals are highly resourceful in the treatment of bacterial ,fungal as well as ameobial infections **.(N. singh *et.al.*,)**

C.rotundus is orally used by people to cure diarrhoea,gastric mucosal injury but no work is carried out on their antimicrobial and antiameobic potential .If these plants showed better results agaist microbes and amoeba ,it will be more beneficial to cure some diseases.

8.PROPOSED WORK PLAN / FORMULATION AND STRUCTURE OF THE STUDY:

Year –wise plan of work: -

1st Year: -

1.Review of Literature.

2.Collection of *Cyperus rotundus* plant.

3.Selection of microbes.

4.Referencing of work.

2nd Year: -

1.Preparation of powder.
2.Preparation of *Cyperus rotundus* plant extracts.
3.Assesment of antimicrobial activity against selected pathogens.
4.Assesment of Antiamoebic activity against selected species of *Amoeba*.
5. Phytochemical analysis of *Cyperus rotundus.*

3rd Year: -

1.Cytotoxicity screening of *C. rotundus*
2.Statistical study of all approaches.
3.Completion of research work.
4.Submission of thesis.

9. EXPECTED OUTCOME

Nowadays, people are focused on herbal medicines. They are blindly using medicinal plants to chronic diseases by listening to the Ramdev Baba and reading the usage of medicinal plants on multimedia. Every plant has hundreds of phytoconstituents which are harmful to the body and may cause different type of side-effects. Research on phytoconstituents and cytotoxicity of these selected plant extracts, therefore, will be crucial in usage of the ethno medicines more scientifically.

It will be more beneficial to the society if herbal remedy is found to cure microbial infections and Ameobiasis and will be helpful to avoid cytotoxicity if any crude extract and other side effects.

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